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13. ABSTRACT *(Maximum 200 Words)*

The neurotoxin 1-methyl-4-phenyl-tetrahydropyridine (MPTP) inhibits mitochondrial oxidative phosphorylation and replicates the pattern of neuronal neurodegeneration in Parkinson's disease (PD). Neurons that degenerate in PD develop inclusions containing aggregates of a synaptic protein, alpha synuclein. The purpose of this study is to determine how MPTP and other neurotoxins affect cytoskeletal and synaptic proteins and to study the relationship between oxidative damage and synuclein aggregation. We found that mice develop synuclein and ubiquitin immunoreactive neuronal aggregates 7-10 days after MPTP administration but that aggregation is not evident 4 days after MPTP treatment. Double staining studies show that oxidative markers are present in neurons that develop alpha synuclein aggregates. Treatment of mice with paraquat leads to nigral degeneration and alpha synuclein aggregation that is more prominent than that produced by MPTP. Proteasomal inhibitors, such as lactacystin and epoxomicin, protect mice from the neurotoxic effects of MPTP but paradoxically increase alpha synuclein aggregation. Alpha synuclein knockout mice are resistant to the neurotoxic effects of MPTP and mitochondrial toxins. Preliminary studies suggest that ubiquitin positive aggregate formation is not found in these animals. Our studies provide new insight into the pathogenesis of neuronal degeneration induced by neurotoxins and may lead to novel therapies for PD.

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Introduction

Neurons that degenerate in Parkinson's disease (PD) develop characteristic inclusions called Lewy bodies that contain aggregates of a synaptic protein, alpha synuclein. Overexpression of mutant forms of synuclein associated with familial PD can lead to aggregate formation in both transgenic mice (1) and fruit flies (2). The pattern of neurodegeneration found in Parkinson's disease (PD) can be replicated in some animal species, including primates and mice, by the systemic administration of neurotoxins such as 1-methyl-4-phenyl-tetrahydropyridine (MPTP)(3,4). MPTP inhibits mitochondrial oxidative phosphorylation and causes oxidative injury leading to cell death (3,5). We and others have shown that MPTP can induce synuclein aggregation (1,4). Oxidative stress may be a key factor leading to synuclein aggregation that in turn may lead to further oxidative injury and the induction of neuronal death (6,7). The purpose of this study is to determine how MPTP and other toxins affect cytoskeletal and synaptic proteins and to study the relationship between oxidative damage and the formation of synuclein aggregates within neurons. This annual report updates our progress for the period August 1st 2001-July 31st, 2002. In the first year of our study we showed that both acute and chronic MPTP treatment, which cause nigral dopaminergic neurons to degenerate, are associated with the displacement of alpha synuclein from its normal synaptic location into neuronal cell bodies. Neuronal degeneration was evident with DAT and calbindin immunocytochemistry and glial reaction was evident with GFAP immunocytochemistry. We also found that the redistribution of synuclein is associated with increased ubiquitin immunoreactivity and increased levels of oxidative markers in the substantia nigra and that the redistribution of synuclein does not appear to be associated with changes in distribution of synaptophysin or neurofilament proteins. In the second year of the study continued to make progress in

accomplishing the experiments outlined in the Statement of Work by quantifying MPTP toxicity in three different strains of mice using two different protocols of MPTP administration. Synuclein aggregation was studied using four well-characterized alpha synuclein antibodies. In year three we have made substantial progress in completing the proposed time course and double labeling studies proposed in the approved Statement of Work. In addition, important new opportunities to study the role of synuclein in aggregate formation and neuronal injury have presented themselves by the availability of synuclein knockout mice and by recent reports published in the past year showing that neurotoxins such as paraquat and proteasomal inhibitors such as lactacystin may be more potent that MPTP in inducing aggregate formation in vitro and possibly in vivo. We performed new studies using paraquat and the proteasomal inhibitor epoxomicin based on this important new data. In the final year of the study (year 4, no cost extension) we will follow up on these important new observation and complete data analysis and manuscript preparation.

Body

We proposed two series of experiments in the approved Statement of Work. The first series of experiments were designed to define changes in the distribution and morphology of alpha synuclein immunoreactivity produced by systemic treatment of MPTP in mice. Both the time course of these changes and their relationship to synaptic (synaptophysin) and neurofilament proteins (NF-M) are being studied. The second series of experiments focuses on spatial and temporal relationships between synuclein aggregation and oxidative injury at the cellular level. Patterns of cell death and apoptosis associated with MPTP toxicity are to be determined and related to the changes in synuclein and oxidative damage.

In the first year of funding we completed the first series of experiments (series 1) on 72 adult male C57BL mice treated with intraperitoneal MPTP followed by sacrifice after a 7-10 day survival period. In the second year we further explored the process of synuclein aggregation by testing four unique synuclein antibodies in different mouse strains and we completed the second set of experiments (series 2) on mice treated with intraperitoneal MPTP followed by sacrifice after a 7-10 day survival period. Quantitative analysis using all antibodies showed a striking increase in synuclein positive cell bodies after MPTP treatment. Similar changes are seen with ubiquitin immunocytochemistry. A few ubiquitin positive cellular profiles are seen in the control substantia nigra. In the acute and chronic MPTP lesions there is a clear increase in the number of ubiquitin positive profiles. In contrast to the striking changes seen with alpha synuclein and ubiquitin staining, the staining pattern of synaptophysin, a synaptic protein, and neurofilament (medium chain), a marker of cell bodies and dendrites, changes minimally. A series of 40 MPTP-treated mice were studied for evidence of oxidative injury using markers such as 8-hydroxydeoxyguanosine, a marker of DNA oxidation, which was clearly increased in neurons in the substantia nigra of MPTP-treated animals after 7-10 day survivals. In year three we have made substantial progress in completing the proposed time course and double labeling studies proposed in the approved Statement of Work. In addition, important new opportunities to study the role of synuclein in aggregate formation and neuronal injury have presented themselves by the availability of synuclein knockout mice and by the discovery that other neurotoxins may be more potent that MPTP in inducing aggregate formation in vivo.

We previously found in our year 2 studies that the severity of MPTP lesions varies from animal to animal and differed between strains. C57BL6 mice are more resistant to the effects of MPTP toxicity than B6CBA mice and lesion extent is smaller and more variable. B6SJL mice

show a greater mean reduction in DAT-positive neurons and less variability within individual groups and so were used in subsequent experiments. In addition we tested two protocols of MPTP administration (acute (higher doses over 2 days) and chronic (lower doses over 10 days). Since both protocols resulted in similar lesions that acute model was used in year 3 studies. As in our previous studies, we defined the extent of MPTP-induced neurodegeneration Immunocytochemically using a monoclonal antibody against the dopamine transporter (DAT). In MPTP treated mice there is a clear reduction in the intensity of immunoreactivity in the striatum that is more severe in the caudal and dorsal aspects of the striatum. There is also depletion of neurons in the substantia nigra, especially in the middle third of the nigra (A8 field) with relative sparing of the medial ventral tegmental area (A10). Individual DAT positive neurons show dendritic and axonal pruning and fragmentation and distortion of immunoreactive processes.

As shown in the appendix illustrations, we performed double labeling studies as proposed in the statement of work. As expected we found that the oxidative markers colocalized in neurons with aggregation formation. Time course data surprisingly we did not see neuronal loss at 4 days post last MPTP dose despite clear loss at 7-10 days.

New reports published in the past year indicate that neurotoxins other than MPTP may also lead to aggregate formation (8, 9). We tested paraquat and the proteasomal inhibitors lactacystin and epoxomicin (10). Paraquat produces a similar degree of nigral degeneration as MPTP but with more robust aggregate formation (see appendix figure). Both lactacystin and epoxomicin reduced MPTP toxicity. Paradoxically they may both be associated with increased synuclein aggregation but further studies will be needed to make a more definitive statement on this issue.

We performed a series of studies using alpha synuclein knockout mice, an important new animal model that was not available when this proposal was initially funded (11). With these animals we can directly test the role of synuclein in aggregate formation and the role of synuclein in MPTP neurotoxicity. We found that these animals are less sensitive to MPTP (draft manuscript enclosed). Baseline histological studies show that they are no different from wild type (11). There was no clear aggregate formation in these animals as defined by ubiquitin staining but further studies are needed to define potential changes.

We have requested a 4th year of the study as a no-cost extension to follow up on these exciting preliminary data and to complete data analysis and manuscript preparation based on our experimental results.

Key Research Accomplishments:

1) Neurons exposed to toxins that induce alpha synuclein aggregation show evidence of oxidative damage. For example, 8-OHDG immunoreactivity, which labels oxidized DNA, is colocalized with alpha synuclein in degenerating neurons within the substantia nigra of MPTP-treated mice.
2) Time course studies suggest that alpha synuclein aggregation is a relatively late phenomenon after MPTP treatment. Preliminary studies show that alpha synuclein immunoreactive aggregates are detected at 7-10 days post MPTP exposure but not at 4 days post exposure.
3) Treatment of mice in vivo with paraquat induces nigral degeneration and alpha synuclein aggregation that is more prominent than that produced by MPTP.

4) Proteasomal inhibitors such as lactacystin and epoxomicin protect mice from the neurotoxic effects of MPTP but paradoxically may lead to increased alpha synuclein aggregation in nigral neurons.

5) Alpha synuclein knockout mice resist the neurotoxic effects of MPTP and mitochondrial toxins. Preliminary studies suggest that ubiquitin positive aggregate formation is not found in these animals in contrast to wild type animals.

Reportable Outcomes

1) Two manuscripts are being prepared and will be submitted for publication. One manuscript is near completion and a draft is enclosed (see appendix materials).
2) The database of histological materials has been further expanded with our new studies and dozens of specimens have been added to our tissue bank and catalogued for future research.
3) Three postdoctoral fellows and three technicians have been trained in surgical and histological procedures and have gained experience in the laboratory supported by this award

Conclusions

MPTP treated mice develop alpha synuclein aggregates in degenerating neurons in the substantia nigra 7-10 days after MPTP administration. Time course studies suggest that aggregate formation is not present 4 days after MPTP administration. The neurodegenerative process is associated with increased levels of oxidative markers that colocalize in neurons that contain alpha synuclein immunoreactivity. Paraquat treatment also induces prominent nigral degeneration and alpha synuclein aggregation that is more prominent than that produced by

MPTP. Proteasomal inhibitors such as lactacystin and epoxomicin protect mice from the neurotoxic effects of MPTP but paradoxically may lead to increased alpha synuclein aggregation in nigral neurons. Alpha synuclein knockout mice resist the neurotoxic effects of MPTP and mitochondrial toxins. Preliminary studies suggest that ubiquitin positive aggregate formation is not found in these animals in contrast to wild type animals. Our new findings provide new insights into the pathogenesis of neuronal degeneration induced by neurotoxins and suggest that therapeutic strategies targeted at interfering with synuclein aggregation may lead to novel therapeutic approaches to the treatment of PD. In the final year of the study (year 4) we will follow up on these important new observation and complete data analysis and manuscript preparation.

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Appendices

Manuscript (draft)

1) Klivenyi P, Ferrante RJ, Giardian G, Kowall NW, Abelovich A, Beal MF. *Mice Lacking Alpha-Synuclein Are Resistant To Mitochondrial Toxins*. In preparation

Color Photographs (set of 3):

Figure legends

Figure 1. Low power photomicrographs of the neostriatum of wild type (A) and alpha synuclein knockout mice (B), using dopamine transporter (DAT) antibody, in MPTP lesioned mice. There is a reduction in DAT immunoreactivity in the wild type mouse with relative protection of DAT immunoreactivity in the alpha synuclein mouse. This reflects the relative preservation of dopamine neurons in the substantia nigra in the MPTP-lesioned alpha synuclein mice.

Figure 2. Substantia nigra in paraquat-treated mice. Alpha synuclein (A) and ubiquitin (B) immunoreactivity in the substantia nigra of paraquat-treated mice. Both intense cellular and neuropil aggregates are observed in the substantia nigra with both antibodies.

Figure 3. Combined alpha synuclein and 3-nitrotyrosine immunoflourescence in MPTP-treated mice. Combined immunoflourescence for alpha synuclein (red) (A) and 3-nitrotyrosine (green) (B) immunoreactivity within the same tissue specimen from the substantia nigra of an MPTP-treated mouse show colocalization of alpha synuclein and 3-nitrotyrosine immunostaining in the merged figure (yellow) (C).

MICE LACKING ALPHA-SYNUCLEIN ARE RESISTANT TO MITOCHONDRIAL TOXINS

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Abstract

Abnormalities in the function of α-synuclein are implicated in the pathogenesis of Parkinson's disease (PD). We found that α-synuclein deficient mice are resistant to MPTP-induced degeneration of dopaminergic neurons. These effects were not due to alterations in MPTP processing, MPP^+ uptake or vesicular transport. We found that α-synuclein deficient mice are also resistant to both malonate and 3-nitropropionic acid (3-NP) neurotoxicity. There was reduced generation of reactive oxygen species in α-synuclein mice following intrastriatal administration of malonate, and reduced histopathologic evidence of oxidative damage following MPTP, 3-NP and malonate. These findings implicate α-synuclein as a modulator of oxidative damage, which has been implicated in neuronal death produced by MPTP and other mitochondrial toxins.

Introduction

A role of α-synuclein in the pathophysiology of Parkinson's disease (PD) has been under intense investigation following the finding that mutations in α-synuclein are associated with dominantly inherited PD, and that α-synuclein appears to the most abundant protein in Lewy bodies, the proteinaceous cytoplasmic inclusions which are the pathologic hallmark of PD and dementia with Lewy bodies (Polymeropoulos et al., 1997; Spillantini et al., 1998; Kruger et al., 1999). α-Synuclein also is part of glial cytoplasmic inclusions of multiple system atrophy (Tu et al., 1998). It is associated with the neuronal intranuclear inclusions of Huntington's disease (HD), and promotes huntingtin aggregation (Furlong et al., 2000; Mezey et al., 2000). Over expression of both wild-type and mutated α-synuclein produced neurotoxicity in drosophilia, mice and rats (Feany et al., 2000; Kirik et al., 2002; Richfield et al., 2002).

The normal physiologic role of α-synuclein is unknown. α-Synuclein is widely expressed in the nervous system, where it is found in presynaptic nerve terminals closely associated with presynaptic vesicles (Goedert, 2001; Cole et al., 2002). However, immunoelectron microscopy, as well as cell-fractionation studies, suggest that synuclein is not stably associated with synaptic membranes (Clayton et al., 1999; Kahle et al., 2000). α-Synuclein however undergoes a marked conformational change upon binding to cellular membranes, and interacts with a number of vesicle-related and microtubule associated molecules (Goedert, 2001). In the substantia nigra dopaminergic neurons α-synuclein may regulate the rate of refilling of releasable pool of synaptic vesicles (Abeliovich et al., 2000).

The neurotoxicity of α-synuclein may be related to its fibrillization. Although both PD α-synuclein mutations [Ala^{53} — Thr (A53T) and Ala^{36} — Pro (A30P)] accelerate the formation of nonfibrillar oligomeric protofibrils in vitro, but A30P inhibits the conversion of protofibrils to

fibrils (Conway et al., 2000). More recently it was shown that dopamine is oxidatively linked to α-synuclein and this prevents the protofibril-to-fibril conversion, causing accumulation of the α-synuclein protofibril (Conway et al., 2001).

Other evidence showed that oxidative damage can cross-link α-synuclein with the formation of dityrosine, or that α-synuclein can be nitrated (Souza et al., 2000; Paxinou et al., 2001). Lewy bodies are nitrated in PD, suggesting that peroxynitrite mediated oxidative damage may contribute to disease pathogenesis (Giasson et al., 2000). α-Synuclein increases oxidative damage in vitro, and sensitizes cells to oxidative insults (Hsu et al., 2000; Ko et al., 2000; Ostrerova-Golts et al., 2000; Lee et al., 2001b).

In order to further explore the function of α-synuclein in neurotoxicity, we examined whether a deficiency of α-synuclein alters susceptibility to mitochondrial toxins. We examined the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin which produces an animal model of PD in α-synuclein deficient mice (Abeliovich et al., 2000). We also examined the susceptibility of these mice to the mitochondrial toxins malonate and 3-NP, which produce striatal toxicity which closely mimic HD.

Discussion

In the present experiments we examined the susceptibility of α-synuclein deficient mice to the neurotoxin MPTP, which has been used to model PD in mice (Beal, 2000). We found that α-synuclein mice are resistant to both dopamine depletion and loss of tyrosine hydroxylase immunostained neurons in the substantia nigra pars compacta. This resistance did not appear to be due to altered uptake or processing of MPTP, since we found no significant differences in MPP^+ levels in the mutant as compared to control mice. Although we found increases in vesicular transport we found no changes in Complex I activity, which is inhibited by MPP^+ in

mitochondria (Takahashi et al., 1997). Sequestration of MPP^+ into vesicles is therefore unlikely to account for the protection we observed.

In the present experiments we also examined the susceptibility of α-synuclein deficient mice to the mitochondrial toxins malonate and 3-NP. Malonate and 3-NP are respectively reversible and irreversible inhibitors of succinate dehydrogenase, which replicate many of the characteristics of pathologic and phenotypic features of HD (Beal et al., 1993; Brouillet et al., 1995). We found that the striatal lesions produced by both of these toxins were significantly decreased in α-synuclein deficient as compared to control mice.

We also examined whether the α-synuclein deficient mice are resistant to oxidative stress. We previously showed that malonate increases hydroxyl radical generation, and that malonate induced striatal lesions are significantly attenuated by free radical scavengers (Schulz et al., 1995). Furthermore malonate lesions are exacerbated in mice deficient in free radical scavenging enzymes (Klivenyi et al., 2000; Andreassen et al., 2001). In the present experiments we found that α-synuclein deficient mice show reduced hydroxyl radical generation following intrastriatal administration of malonate, consistent with the neuroprotective effects seen in mice.

How might α-synuclein modulate oxidative damage. α-Synuclein appears to be important in vesicular loading of dopamine (Abeliovich et al., 2000). A recent study showed reduced MPTP induced release of dopamine was reduced in another line of α-synuclein deficient mice (Dauer et al., 2001). When α-synuclein expression is reduced in cultured rat neurons the number of vesicles in the distal pool of the presynpatic terminal is reduced (Murphy et al., 2000). A reduction in dopamine release may reduce the generation of free radicals produced by monoamine oxidase metabolism, or autooxidation of dopamine itself (Sulzer et al., 2000). The importance of cytoplasmic dopamine to PD cell death is supported by the finding that the

dopaminergic neurons of the ventral tegmental area, which are resistant as compared to the substantia nigra, express high levels of the VMAT, which promotes vesicular sequestration of dopamine, and low levels of the dopamine transporter which pumps dopamine into the cytoplasm (Takahashi et al., 1997). Furthermore neuromelanin containing neurons, which are a polymerization product of dopamine-derived orthoquinone, are relatively sensitive to cell death in PD (Sulzer et al., 2000).

If reduced dopamine release contributes to neuroprotection in the α-synuclein deficient mice, this may help to explain the paradox that transgenic mice overexpressing wild-type or mutant α-synuclein do not show increased vulnerability to MPTP (Rathke-Hartlieb et al., 2001). In these mice increased α-synuclein expression above a threshold may not alter vesicular release of dopamine. In transgenic mice which overexpress either wild-type mutated α-synuclein under a tyrosine hydroxylase promotor however there is an increase in the dopamine transporter and enhanced vulnerability to MPTP (Richfield et al., 2002). This is consistent with other evidence that there is direct and functional binding of α-synuclein to the dopamine transporters to accelerate dopamine induced apoptosis (Lee et al., 2001a). A reduction in vesicular release of dopamine may also explain the neuroprotection seen in the α-synuclein mice against both malonate and 3-NP neurotoxicity. Striatal lesions produced by malonate and 3-NP, as well as the generation of reactive oxygen species, are significantly attenuated in rats with 6-hdyroxydopamine lesions of the striatum, or pharmacologic depletion of dopamine (Maragos et al., 1998; Reynolds et al., 1998; Xia et al., 2001). Furthermore systemic or intrastriatal administration of L-DOPA or dopamine, respectively, restores malonate toxicity and generation of reactive oxygen species in 6-hydroxydopamine lesioned rats (Xia et al., 2001).

The present findings are consistent with a role of α-synuclein in modulating dopamine release and oxidative damage in PD. There may be a complex interaction as suggested by the observation that oxidative forms of dopamine can promote α-synuclein protofibril generation (Conway et al., 2001). Expression of mutant α-synuclein causes increased susceptibility to dopamine toxicity, and an α-synuclein fragment produces neurotoxicity to dopaminergic neurons both in vitro and in vivo (Forloni et al., 2000; Tabrizi et al., 2000). Furthermore administration of rotenone, a selective mitochondrial complex I inhibitor which generates ROS, can produce, selective damage to substantia nigra neurons, and α-synuclein positive Lewy bodies (Betarbet et al., 2000). MPTP can upregulate α-synuclein expression in both mice and primates (Kowall et al., 2000; Vila et al., 2000). Other environmental toxins which produce oxidative damage and are implicated PD pathogenesis also upregulate α-synuclein (Manning-Bog et al., 2002). Lastly oxidative damage may contribute to Lewy body generation (Giasson et al., 2000). The mutations in α-synuclein in familial PD may promote the ability of α-synuclein to generate protofibrils. In sporadic PD exposure to environmental toxins may produce oxidative damage, and promote α-synuclein expression and aggregation, which is then exacerbated by dopamine.

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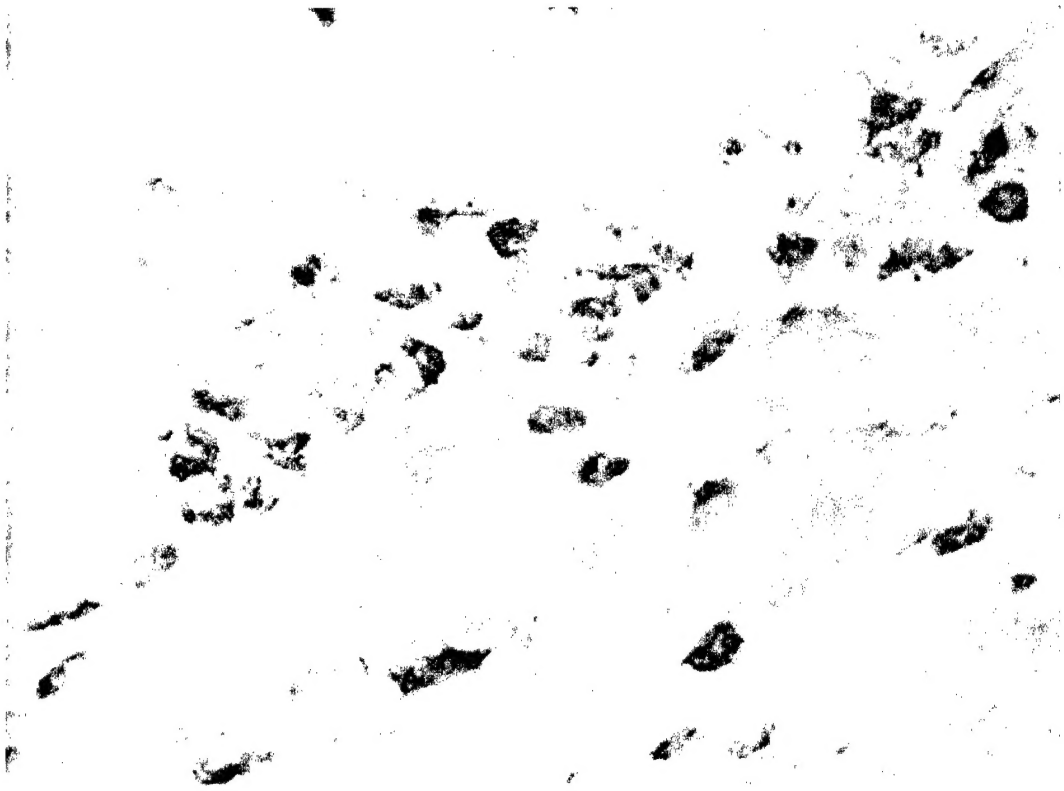
Figure 1 - Page 27



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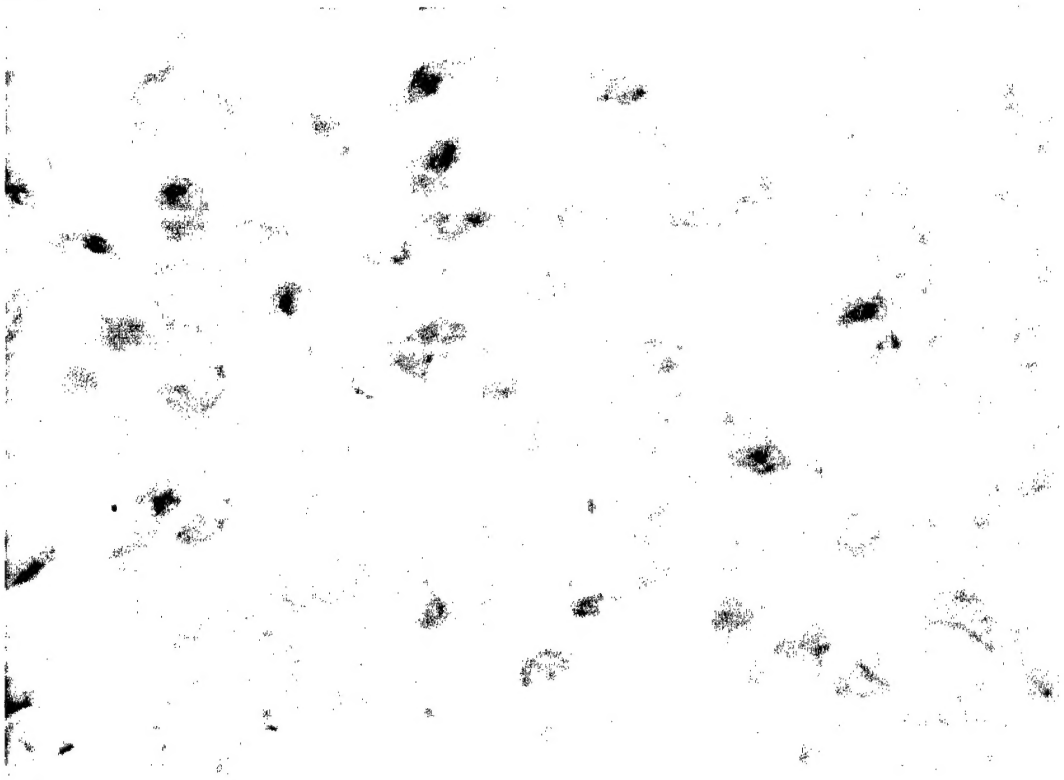

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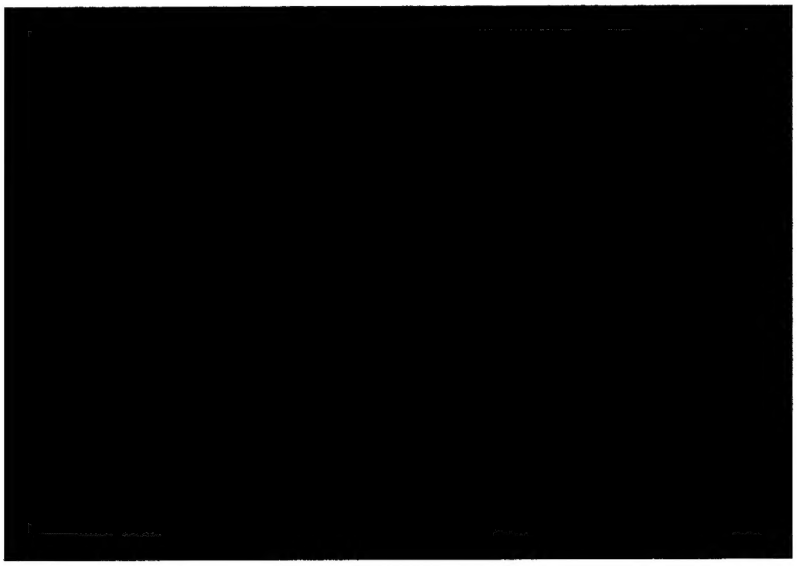

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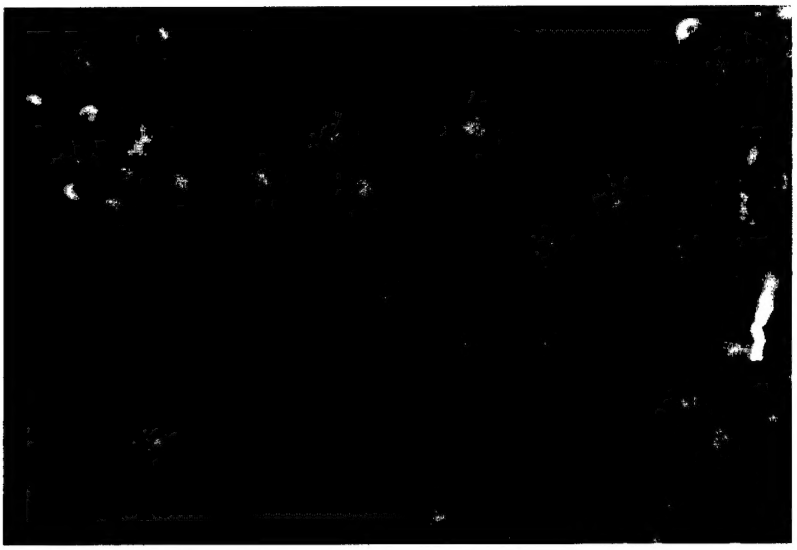

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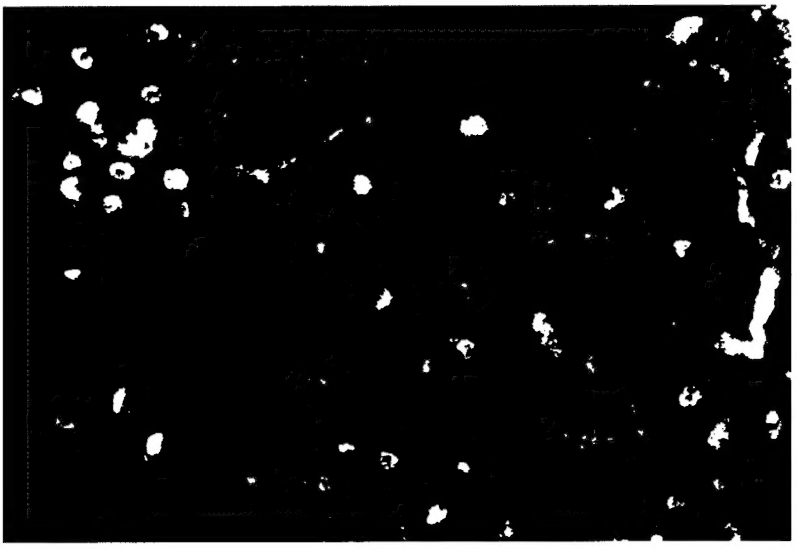

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